The Plant Journal (1996) 9(4), 431–439

Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein

N.E. Strobel[1], C. Ji[1], S. Gopalan[2], J.A. Kuc[1] and S.Y. He[1,2,*]
[1]*Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA, and*
[2]*MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312, USA*

Summary

Systemic acquired resistance (SAR) is an inducible plant defense response and is effective against a broad spectrum of pathogens. Biological induction of SAR usually follows plant cell death resulting from the plant hypersensitive response (HR) elicited by an avirulent pathogen or from disease necrosis caused by a virulent pathogen. The elicitation of the HR and disease necroses by pathogenic bacteria is controlled by *hrp* genes. Previously, it was shown that the *Pseudomonas syringae* 61 (Pss61) $HrpZ_{Pss}$ protein (formally $harpin_{Pss}$) elicited the HR in plants. In this study, it is shown that $HrpZ_{Pss}$ induced SAR in cucumber to diverse pathogens, including the anthracnose fungus (*Colletotrichum lagenarium*), tobacco necrosis virus and the bacterial angular leaf spot bacterium (*P. s.* pv. *lachrymans*). A *hrpH* mutant of Pss61, which is defective in the secretion of $HrpZ_{Pss}$ and, possibly, other protein elicitors, failed to elicit SAR. Pathogenesis-related (PR) proteins, including peroxidase, β-glucanase and chitinases, were induced in cucumber plants inoculated with Pss61, *C. lagenarium* or $HrpZ_{Pss}$. The induction patterns of PR proteins by $HrpZ_{Pss}$ and Pss61 were the same, but were different from that induced by *C. lagenarium*. Interestingly, the *hrpH* mutant induced two of the three identified PR proteins, despite its failure to induce SAR. These results suggest that proteinaceous elicitors, such as $HrpZ_{Pss}$, that traverse the bacterial Hrp secretion pathway are involved in the biological induction of SAR and that at least some PR proteins can be induced by bacterial factors that are not controlled by *hrp* genes.

Introduction

Localized infection of plants by necrotizing pathogens can result in systemic acquired resistance (SAR) to disease, which persists for weeks to months and is effective against diverse pathogens including fungi, bacteria, and necrotizing viruses (Kuc, 1982; Ross, 1961). Biological induction of SAR is usually associated with prior plant cell death during the hypersensitive response (HR) or disease necrosis triggered by avirulent or virulent pathogens, respectively (Cameron *et al.*, 1994; Kuc, 1982; Ross, 1961; Uknes *et al.*, 1993). Certain synthetic chemicals, such as salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), also can be very effective in the induction of SAR when applied to plants (Metraux *et al.*, 1991; White, 1979). The induction of SAR in cucumber plants by an avirulent bacterial pathogen, *Pseudomonas syringae* pv. *syringae*, appears to be dependent on bacterial *hrp* genes that are required for many plant pathogenic bacteria to elicit the HR in non-host plants or to cause disease in host plants (Smith *et al.*, 1991). The HR is a complex plant resistance reaction which involves local plant cell death and restriction of pathogens to the site of their introduction (Klement, 1982).

Recent studies have shown that most Hrp proteins are involved in the assembly of a type III protein secretion pathway (the Hrp pathway) through which bacterial pathogenesis-related proteins traverse to the extracellular milieu to initiate various plant–bacterial interactions (Fenselau, 1992; Huang *et al.*, 1992, 1995; Van Gijsegem *et al.*, 1995). One family of such proteins that have been identified are heat-stable, glycine-rich proteins: harpin of *Erwinia amylovora* (Wei *et al.*, 1992), $HrpZ_{Pss}$ (formally $harpin_{Pss}$) of *P. s.* pv. *syringae* 61 (Pss61) (He *et al.*, 1993) and PopA of *P. solanacearum* (Arlat *et al.*, 1994). Harpins and PopA were shown to elicit the HR when infiltrated into the leaf laminae of appropriate plants (Arlat *et al.*, 1994; He *et al.*, 1993; Wei *et al.*, 1992), to induce exchange of H^+ and K^+ (the 'XR') across the plasmalemma (Wei *et al.*, 1992), and to generate active oxygen species (Baker *et al.*, 1993) when added to plant cell cultures, which are all properties of the HR elicited by live bacteria.

As part of our investigation into plant responses to *P. syringae* extracellular proteins under the control of the Hrp regulatory/secretion system, we studied the involvement of $HrpZ_{Pss}$ in the biological induction of SAR by *P. s.* pv. *syringae* 61. In this paper we describe the experimental results showing that $HrpZ_{Pss}$, as well as the bacterium (Pss61) that produces it, efficiently induced SAR in cucumber to diverse pathogens, including a fungus (*Colletotrichum lagenarium*), a bacterium (*P. s.* pv. *lachrymans*) and a local lesion-forming virus (tobacco necrosis virus). The *hrpH* mutant, which is defective in the secretion of $HrpZ_{Pss}$, failed to induce SAR. Multiple pathogenesis-related (PR) proteins were detected in cucumber plants treated with $HrpZ_{Pss}$, Pss61 and *C. lagenarium*. The efficacy

Received 19 September 1995; revised 2 January 1996; accepted 9 January 1996.
*For correspondence (fax +1 517 353 9168; e-mail hes@pilot.msu.edu).

of SAR induction, resistance spectrum and patterns f PR protein induction were very similar in plants treated with $HrpZ_{Pss}$ and Pss61. Interestingly, the PR protein patterns induced by $HrpZ_{Pss}$ and Pss61 were somewhat different from that induced by *C. lagenarium*. The *hrpH* mutant, though unable to induce SAR, efficiently induced some of the well-characterized PR proteins. These results suggest that the biological induction of SAR by *P. syringae* is dependent on the bacterial proteins (such as $HrpZ_{Pss}$) which traverse the Hrp secretion pathway and that at least some PR-proteins can be induced by bacterial factors other than Hrp-controlled extracellular proteins.

Results

Symptoms on cucumber leaves treated with SAR inducers

Treatment of leaves with spores of *C. lagenarium* (a virulent, necrogenic pathogen of cucumber) resulted in the development of symptoms typically obtained with the fungus in cucumber: infiltrated areas were asymptomatic for 3–4 days, after which time tissues began to collapse and become necrotic. Lesions continued to expand for several days and developed a tan to brown pigmentation. Symptoms induced by treatments with Pss61 (an avirulent, HR necrosis-inducing pathogen) and $HrpZ_{Pss}$ varied with environmental conditions in the greenhouse. Under high levels of natural light, Pss61 and $HrpZ_{Pss}$ triggered the HR within 24 and 48 h, respectively, after infiltration. The HR was restricted to infiltrated areas and did not expand as did the necroses caused by *C. lagenarium*. Under lower natural light levels (cloudy days), tissues infiltrated with Pss61 or $HrpZ_{Pss}$ developed a weaker HR characterized by increasing chlorosis over a 3–5 day period, then necroses developed gradually and irregularly, despite supplemental illumination with sodium lamps. Infiltration with *hrpH* (which is defective in the secretion of $HrpZ_{Pss}$, He *et al.*, 1993) caused either no symptoms or a very mild chlorosis under all conditions tested. Infiltration with buffer alone caused only a small ring of white necrosis resulting from mechanical damage caused by pressure of the pipette mouth against the leaf. Interestingly, infiltration with *E. amylovora* harpin protein, which was prepared from DH5α(pCPP50) (He *et al.*, 1994) and which induced a strong HR in tobacco leaves, did not induce HR necrosis in cucumber leaves (data not shown).

SAR to C. lagenarium

We first tested to see whether $HrpZ_{Pss}$ alone could induce SAR to a well-studied fungal pathogen of cucumber, *C. lagenarium*. As shown in Table 1, $HrpZ_{Pss}$ treatment induced SAR comparable to that induced by *C. lagenarium* (approximately 90% reduction in total necrotic area relative to buffer-treated controls) in two upper leaves which expanded subsequent to induction treatment. The degrees of SAR induced by $HrpZ_{Pss}$, Pss61, Pss61-*hrpH* and *C. lagenarium* in cucumber were subsequently compared. Under conditions conducive to HR development in the greenhouse (high levels of natural light due to sunny weather) both $HrpZ_{Pss}$ and Pss61 efficiently induced SAR in Leaf 2 and Leaf 3 (Table 2 and Figure 1a and b). SAR was expressed as a reduction in both the number and diameter of necrotic lesions resulting from challenge with *C. lagenarium*. Protection of Leaf 2 was comparable to that induced by *C. lagenarium*, whereas protection in Leaf 3 was weaker than that induced by the fungus. Under the conditions of this experiment, expansion of Leaf 2 and Leaf 3 occurred after the onset of the HR and necrosis incited by *C. lagenarium* infiltration. Leaf 2 was fully expanded prior to challenge-inoculation, whereas Leaf 3 was not. The *hrpH* mutant did not induce SAR (Table 2). The quality and/or quantity of light profoundly influenced the induction of both the HR and SAR in cucumber by Pss61 and $HrpZ_{Pss}$ in the greenhouse. When a similar experiment was conducted under conditions non-conducive to HR development (low levels of natural light on cloudy days), neither Pss61 nor $HrpZ_{Pss}$ induced the HR or SAR, although *C. lagenarium* incited necrotic lesions on Leaf 1 and induced SAR under these conditions (data not shown).

SAR to TNV

We next examined whether $HrpZ_{Pss}$-induced SAR would be effective against a viral pathogen. In two initial experiments, the abilities of $HrpZ_{Pss}$ and *C. lagenarium* to induce SAR to TNV were compared. $HrpZ_{Pss}$ elicited a normal HR in these experiments and induced SAR to TNV local lesion formation comparable to that induced by *C. lagenarium* (Table 3 and Figure 1c and d). We then compared the abilities of $HrpZ_{Pss}$, Pss61, *hrpH*, and *C. lagenarium* to induce SAR to TNV. Under high light conditions, $HrpZ_{Pss}$ and Pss61 elicited a normal HR and induced SAR which restricted local lesion formation by TNV to an extent similar to that of SAR induced by *C. lagenarium*. The percentage of lesion number reduction was 68% for Pss61, 67.1% for $HrpZ_{Pss}$, and 75.5% for *C. lagenarium* (Table 3). Under low natural light conditions unfavorable for HR development (see Experimental procedures), $HrpZ_{Pss}$ and Pss61 elicited a weaker degree of SAR relative to that induced by *C. lagenarium*. The percentage of lesion number reduction was 44.9% for Pss61, 46.7% for $HrpZ_{Pss}$, and 89.6% for *C. lagenarium* (Table 3). The lesion numbers observed in these independent experiments varied greatly, mainly due to the use of different TNV inoculum preparations. TNV inoculum was prepared freshly each time from cucumber

Table 1. Induction by HrpZ$_{Pss}$ and the fungal pathogen, *C. lagenarium*, of systemic acquired resistance to *C. lagenarium* in cucumber

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	18.8 ± 0.6[a]	2.0 ± 0.1	60.9 ± 7.4	18.5 ± 0.6	2.5 ± 0.3	110.2 ± 29.0
HrpZ$_{Pss}$	6.5 ± 0.9	1.1 ± 0.0	6.9 ± 1.3	9.5 ± 1.7	1.3 ± 0.1	13.4 ± 3.7
C. lagenarium	3.3 ± 0.8	1.0 ± 0.0	2.6 ± 0.8	6.5 ± 1.3	1.2 ± 0.1	7.5 ± 1.3

[a]Mean + SE of four replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), or HrpZ$_{Pss}$ (80 μg ml^{-1}) in buffer, or spores of *C. lagenarium* (5×10^4 spores ml^{-1}). After 7 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

Table 2. Induction of systemic acquired resistance to *C. lagenarium* in cucumber by *P. s.* pv. *syringae* 61 (Pss61), HrpZ$_{Pss}$, the *hrpH* mutant of Pss61 and *C. lagenarium*

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	15.4±1.2[a]	1.6±0.2	38.9±8.3	16.2±1.0	1.8±0.1	52.0±8.1
hrpH	13.2±1.1	1.7±0.1	32.1±2.2	15.4±1.6	1.8±0.1	50.0±11.9
Pss61	5.4±0.4	1.2±0.1	7.0±1.9	9.4±1.1	1.5±0.1	21.2±6.2
HrpZ$_{Pss}$	5.0±0.5	1.2±0.1	5.9±1.4	8.6±2.5	1.6±0.2	24.4±9.1
C. lagenarium	4.0±1.2	1.3±0.3	8.4±5.3	6.4±1.4	1.4±0.2	13.2±5.0

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), bacteria (OD_{600}=0.2), HrpZ$_{Pss}$ (160 μg ml^{-1}), or spores of *C. lagenarium* (5×10^4 ml^{-1}). After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

leaves bearing TNV lesions. In experiment 3, the *hrpH* mutant induced a low level of SAR to TNV (Table 3).

SAR to P. syringae *pv.* lacrymans

HrpZ$_{Pss}$ and *C. lagenarium* also induced SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*. For these experiments, cucumber plants were challenge-inoculated at 11 days (by spraying) or 17 days (by rubbing) after treatment of Leaf 1 (Table 4). Although *C. lagenarium* was a more effective treatment, HrpZ$_{Pss}$ also induced significant levels of SAR to the bacterium, reducing necrotic lesion numbers by 32 and 75%, compared with 50 and 86% for *C. lagenarium*, in the two experiments, respectively.

Induction of PR proteins

PR proteins that accumulated in treated cucumber plants were first analyzed using native polyacrylamide gel electrophoresis (PAGE). All treatments (*C. lagenarium*, Pss61 and HrpZ$_{Pss}$) that induced SAR also induced the accumulation of three PR protein bands (tentatively named PR-A, PR-B and PR-C) (Figure 2a). *C. lagenarium* induced PR-C, but not PR-A and PR-B, in systemic leaves, while Pss61 and HrpZ$_{Pss}$ induced PR-B, but not PR-A and PR-C, in systemic leaves. Treatment with buffer or *hrpH* mutant did not induce these particular PR protein bands to levels that would allow visual identification. To see whether any PR proteins with known functions were induced in these plants, protein extracts were analyzed using native PAGE coupled with enzyme (chitinase, peroxidase and β-glucanase) activity staining. As shown in Figure 2(b), all three enzymes were induced in plants treated with HrpZ$_{Pss}$, Pss61 or *C. lagenarium* in both local (treated) and systemic leaves, although induction of chitinase isoforms by Pss61 and HrpZ$_{Pss}$ in systemic leaves was variable and low. The enzyme activities were substantially higher in local leaves than in systemic leaves. Surprisingly, although the *hrpH* mutant bacterium failed to induce SAR, it efficiently induced peroxidase and chitinase, especially in treated leaves (Figure 2b). Only β-glucanase was not found to be induced to high levels in the *hrpH*-treated plants (Figure 2b). It is interesting to note that PR protein levels induced by various treatments correlated well with degrees of SAR induced by the same treatments (*C. lagenarium* >HrpZ$_{Pss}$=Pss61>*hrpH*> or = buffer).

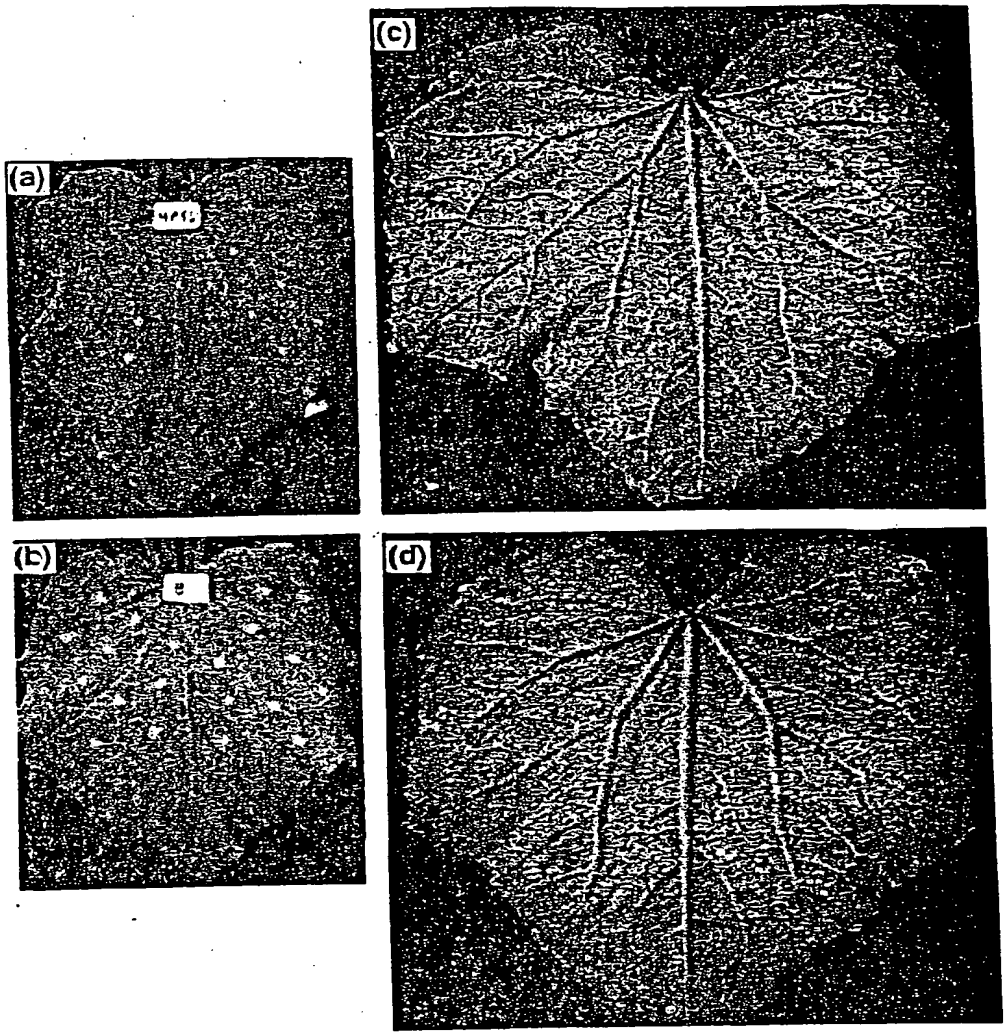

Figure 1. Disease symptoms caused by challenge-infection of *C. lagenarium* and tobacco necrosis virus on cucumber leaves with or without prior induction of SAR.
Anthracnose symptoms on Leaf 2 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (80 μg ml^{-1}, a) or buffer (5 mM $MgSO_4$, b). Leaf 1 of young plants was infiltrated with buffer or $HrpZ_{Pss}$. After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days, when the picture was taken.
TNV symptoms on Leaf 3 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (c) or buffer (d). Leaf 1 was treated by infiltration of buffer or $HrpZ_{Pss}$ as described in footnotes to Table 1. After 7 days, Leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 9 days, when the picture was taken.

Induction of the pr-1 *gene and SAR in tobacco*

$HrpZ_{Pss}$ also induced SAR to tobacco mosaic virus (TMV) in tobacco (Table 5). The SAR level induced by $HrpZ_{Pss}$ was less than that induced by TMV. This was consistent with the different levels of induction of the *pr*-1 gene by $HrpZ_{Pss}$ and TMV (Figure 3). TMV-inoculated local leaves (the third and fourth true leaves) also showed more necrosis than those infiltrated with $HrpZ_{Pss}$ (data not shown), which may be partly responsible for the different levels of SAR and *pr*-1 expression in TMV- and $HrpZ_{Pss}$-induced plants.

Discussion

In this study, we show that $HrpZ_{Pss}$, a bacterial hrp gene product secreted via the Hrp pathway of *P. s.* pv. *syringae*, induced SAR in cucumber and tobacco. In cucumber, the

Table 3. Induction of systemic acquired resistance to TNV in cucumber by *hrpH* mutant, HrpZ$_{Pss}$, Pss61 and *C. lagenarium*

Treatment	Number of TNV necrotic local lesions			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Buffer	99.7±19.6[a]	47.2±0.9[b]	730.0±63.9[a]	342.8±34.3[a]
hrpH	–	–	556.0±53.4	324.3±11.2
HrpZ$_{Pss}$	28.7±3.8	7.5±1.2	240.4±27.5	182.8±18.8
Pss61	–	–	239.9±59.7	189.0±41.9
C. lagenarium	34.7±16.6	9.0±1.8	178.8±26.9	35.8±4.6

[a]Mean ± SE of three replicate plants per treatment. [b]Mean ± SE of eight replicate plants per treatment.
Leaf 1 was treated by infiltration of candidate inducers as described in the footnotes of Table 1. After 7 days, leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 10 or 9 days in experiments 1 and 2, respectively.
Experiments 1, 2 and 3 were performed under high levels of natural light during induction periods.
Experiment 4 was performed on cloudy days.

Table 4. Induction of systemic acquired resistance to *P. syringae* pv. *lacrymans* by HrpZ$_{Pss}$ and *C. lagenarium*

Treatment	Number of necrotic lesions[a]	
	Inoculated by rubbing	Inoculated by spraying
Buffer	244.8±34.2	56.6±5.9
HrpZ$_{Pss}$	168.5±24.5	13.8±1.7
C. lagenarium	122.6±9.8	8.3±2.1

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with treatments as described in the footnotes of Table 1. Leaf 5 was challenged by rubbing, or by spraying the abaxial leaf surface with a suspension of bacterial cells (OD_{600}=0.2, 17 days after induction; or OD_{600}=0.1, 11 days after induction, respectively). Disease was allowed to develop for 7 or 13 days in rub-inoculated or spray-inoculated plants, respectively.

efficacy against fungal, viral and bacterial pathogens and persistence (for at least 17 days in the bacterial challenge experiments) of HrpZ$_{Pss}$-induced SAR is comparable to that induced by the bacterium (Pss61) that produces HrpZ$_{Pss}$. The degree of SAR induced in cucumber by HrpZ$_{Pss}$ was also comparable to that induced by a well-studied biological inducer of SAR, *C. lagenarium* (Kuc and Richmond, 1977). The *hrpH* mutant of *P. s.* pv. *syringae*, which is defective in the secretion of HrpZ$_{Pss}$ and other proteinaceous pathogenicity factors (He *et al.*, 1993; Huang *et al.*, 1992; Yuan *et al.*, in preparation), failed to induce SAR in cucumber. The induced PR protein patterns were the same in cucumber plants treated with Pss61 and HrpZ$_{Pss}$, but were different from that in *C. lagenarium*-treated plants. Moreover, the *hrpH* mutant, although unable to induce SAR, efficiently induced at least two well-characterized PR proteins, chitinase and peroxidase (Figure 2b). These results suggest that the biological induction of SAR and PR proteins by *P. s.* pv. *syringae* 61 in the non-host plant, cucumber, is dependent on the production and secretion of proteinaceous elicitors of the HR, such as HrpZ$_{Pss}$, but that at least some PR proteins can be induced by bacterial molecules independent of *hrp* gene functions.

The efficacy of both HrpZ$_{Pss}$ and Pss61 as inducers of SAR in cucumber appeared to be contingent upon their ability to elicit a normal HR, as low levels of natural light during the induction period, which interfered with HR development, resulted in reduced SAR to TNV and no SAR to *C. lagenarium* (Table 3; Strobel and He, unpublished work). The negative effect of low light likely resulted from an effect on HR development rather than upon the plant's capacity to express SAR because *C. lagenarium* formed necrotic lesions typical of this compatible pathogen on Leaf 1 (the inducer leaf) and triggered SAR under these same conditions. The profound effect of light on the development of the HR has been observed previously (Sequeira, 1979), although the underlying mechanism remains to be determined. The dependence of the induction of SAR on the HR is further suggested by our observations that the *hrpH* mutant of Pss61, which produces but does not secrete HR elicitors (He *et al.*, 1993), did not elicit the HR or induce SAR in cucumber. Furthermore, *E. amylovora* harpin, another HR elicitor which is structurally different from HrpZ$_{Pss}$ and which elicited a strong HR in tobacco, did not induce an HR or SAR in cucumber plants (Strobel and He, unpublished observation). In conclusion, there appears to be a tight linkage between HR development and induction of SAR in plants by avirulent bacteria.

The tight linkage between the HR and SAR suggests that the signal(s) for the induction of SAR by HrpZ$_{Pss}$ and *P. s.* pv. *syringae* 61 likely comes from dying plant cells and/or cells immediately adjacent to the dying cells during the HR. What types of cell death would lead to the induction of SAR? It has been shown that the HR triggered by live bacteria (Keen *et al.*, 1981), HrpZ$_{Pss}$ (He *et al.*, 1993) or *E. amylovora* harpin (He *et al.*, 1994) involves an active cell death pathway. Does this mean that only cells undergoing active cell death give rise to signals for SAR? The answer to this is probably not simple. SAR and PR proteins can

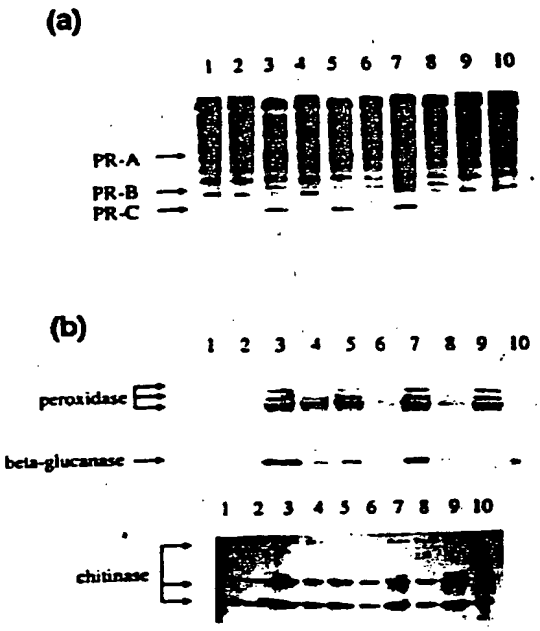

Figure 2. PR protein accumulation in cucumber plants.
PAGE (a) and PAGE coupled with activity staining (b) analyses of protein extracts from treated (lanes 1, 3, 5, 7 and 9) or systemic leaves (lanes 2, 4, 6, 8, and 10). The treatments were buffer (lanes 1 and 2), *C. lagenarium* (lanes 3 and 4), Pss61 (lanes 5 and 6), $HrpZ_{Pss}$ (lanes 7 and 8) and the *hrpH* mutant (lanes 9 and 10). PR-A, PR-B and PR-C are tentative names for the three PR proteins observed in these experiments. The identities of these PR proteins are unknown.

be induced not only by HR-eliciting avirulent pathogens, but also by necrosis-causing virulent pathogens. For example, *P. s.* pv. *lacrymans* and *C. lagenarium* can efficiently induce SAR and/or PR proteins in the susceptible host plant, cucumber (Kuc and Richmond, 1977; Smith *et al.*, 1991; this study). Unless cell death during the HR and some diseases shares the same biochemical processes, which is possible, the ability of both virulent and avirulent pathogens to induce SAR argues for multiple cell death pathways in the induction of SAR. On the other hand, not all types of plant cell death induce SAR. For example, cell death due to mechanical wounding or resulting from certain plant mutations does not induce SAR (Dietrich *et al.*, 1994). It would be important in the future to learn why certain cell death processes, but not others, lead to SAR. Endogenous signaling molecules, such as salicylic acid and H_2O_2, have been shown or suggested to be involved in the induction of SAR (Chen *et al.*, 1993; Gatfney *et al.*, 1993; Malamy *et al.*, 1990; Metraux *et al.*, 1990; Rasmussen *et al.*, 1991). However, the mechanism(s) by which various biological inducers of SAR generate these signals and the identity of the actual systemic signal(s) translocated from the induced leaves to distant leaves remain to be determined. Also, it has not been unequivocally shown that cell death is necessary for the induction of SAR.

Table 5. Induction of systemic acquired resistance to TMV by $HrpZ_{Pss}$ and TMV

	Diameter of necrotic lesions[a]
Buffer	4.41±0.05
$HrpZ_{Pss}$	3.05±0.03
TMV	2.34±0.03

[a]Mean ± SE of 100 lesions per treatment.
The third and fourth true leaves of 6-week-old tobacco plants were inoculated with TMV (100–150 lesions per leaf), or infiltrated with 120 μg ml^{-1} $harpin_{Pss}$ or 5 mM $MgSO_4$ at 10 sites (50 μl per site). Five days later the seventh and eighth true leaves were challenge-inoculated with TMV. The diameters of TMV lesions on the challenged leaves were recorded.

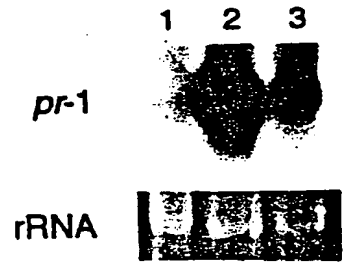

Figure 3. Induction of the *pr*-1 gene in tobacco leaves.
Total RNA was isolated from systemic leaves (the ninth true leaves) of plants treated with buffer (lane 1), TMV (lane 2), or $HrpZ_{Pss}$ (lane 3) 5 days post-induction. A PCR-amplified internal fragment of the tobacco *pr*-1 gene was labeled with [α-^{32}P]dATP and used as a probe. The largest rRNA species visualized after staining with ethidium bromide was used as a reference.

It is interesting to observe that, although *C. lagenarium* (a necrotizing pathogen of cucumber), Pss61 (an HR-eliciting bacterium on cucumber) and $HrpZ_{Pss}$ (an HR-eliciting protein) all induced SAR in cucumber plants, there were some differences in the induction of PR proteins by these pathogens/protein. While *C. lagenarium*, Pss61 and $HrpZ_{Pss}$ all induced PR-A, PR-B and PR-C in the inoculated leaves, only *C. lagenarium* induced PR-C in systemic leaves to a high level (visible on a PAGE gel). In contrast, PR-B was induced in systemic leaves to high levels only by $HrpZ_{Pss}$ and Pss61. The induction patterns of PR-A, PR-B, PR-C, chitinase, peroxidase and β-glucanase were the same for Pss61 and $HrpZ_{Pss}$, suggesting that $HrpZ_{Pss}$ either is a major inducer of SAR in Pss61 or is representative of SAR inducers produced by Pss61. The differences in the induction of PR proteins by *C. lagenarium* and Pss61/$HrpZ_{Pss}$ may have resulted from different inducers produced by *C. lagenarium* and Pss61/$HrpZ_{Pss}$, respectively. Alternatively, the differences may reflect possible mechan-

istic differences of plant cell death resulting from the HR caused by Pss61 or $HrpZ_{Pss}$ and disease necrosis caused by *C. lagenarium*, respectively, alth ugh both types of cell death efficiently trigger SAR in cucumber.

In this study, 80–160 μg ml^{-1} purified $HrpZ_{Pss}$ were used for induction of SAR. $HrpZ_{Pss}$ at these concentrations consistently elicited both HR and SAR in cucumber and tobacco leaves. It is not known whether these concentrations are comparable to the *in vivo* amounts of $HrpZ_{Pss}$ secreted by Pss61. Nor is it known whether the relative activity of purified $HrpZ_{Pss}$ is comparable to that of $HrpZ_{Pss}$ produced by Pss61 *in planta*. Previously, it was shown that Pss61 *hrpZ* mutants carrying transposon-induced mutations in the *hrpZ* gene (complementation group XII) were defective in the elicitation of HR (Huang *et al.*, 1991) and SAR (data not shown). More recently, it was discovered that these transposon-induced *hrpZ* mutations exert a polar effect on five downstream *hrp* genes (*hrpB–F*) in the *hrpZ* operon (Preston *et al.*, 1995; Collmer, personal communication). *hrpB–F*, like *hrpH*, are likely involved in the assembly of the Hrp secretion apparatus (Preston *et al.*, 1995). Therefore, current *hrpZ* mutations affect the expression of not only the *hrpZ* gene but also several other *hrp* genes that are involved in the secretion of $HrpZ_{Pss}$ and, most likely, other HR elicitors/pathogenicity factors. A non-polar *hrpZ* mutant is needed to assess the contribution of $HrpZ_{Pss}$ in the induction of HR and SAR. Recently, several additional proteins traversing the *P. syringae* Hrp secretion pathway have been identified in *P. syringae* pv. *tomato* (Yuan *et al.*, in preparation). It would be interesting to know whether some of these new Hrp-controlled *P. syringae* extracellular proteins can elicit HR and/or SAR.

Although the *hrpH* mutant of Pss61 failed to induce SAR in most experiments, it efficiently induced the accumulation of peroxidase and chitinase in all experiments (Figure 2b and data not shown). The induction of chitinase by *hrp* mutants was also observed by Jakobek and Lindgren (1993). These data suggest that induction of PR proteins is not necessarily a reflection of induction of SAR and that the accumulation of certain PR proteins may not contribute to resistance. In our experiments, only the accumulation of β-glucanase seemed to correlate with the SAR induced by both *C. lagenarium* and Pss61/$HrpZ_{Pss}$ in cucumber. None of the other identified PR proteins were present at high levels in systemic leaves of all cucumber plants that exhibited SAR. Whether β-glucanase is responsible for the resistance of the induced plants to *C. lagenarium*, TNV and *P. s.* pv. *lacrymans* in cucumber remains to be investigated. The relationships between the PR-A, PR-B, and PR-C proteins with β-glucanase, chitinase, or peroxidase are not known.

The demonstration of $HrpZ_{Pss}$ as a proteinaceous inducer of SAR may have important practical implications for plant disease management. Crop plants could be genetically engineered with genes encoding proteinaceous HR/SAR inducers, such as $HrpZ_{Pss}$, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR and SAR would be triggered in otherwise compatible interactions, limiting the disease development.

Experimental procedures

Growth of plants

Cucumber (*Cucumis sativus* L.) plants were grown in plastic pots containing Promix soil. A liquid fertilizer (Peter's 15-16-17, W. R. Grace and Co., Fogelsville, PA), containing 110 p.p.m. nitrogen, was supplied to the water, beginning when the first true leaf was fully open. Plants were grown in a glass greenhouse equipped with high-pressure sodium lights (with a photoperiod of 14 h) to supplement sunlight when necessary.

Preparation of inocula

$HrpZ_{Pss}$ was purified by affinity chromatography from *Escherichia coli* DH5α(pSYH45). pSYH45 is a derivative of pQE30 (Qiagen, Inc.) expressing a hexahistidine–$HrpZ_{Pss}$ (full-length) fusion protein. The first methionine residue of $HrpZ_{Pss}$ was replaced by the following amino acid sequence in the fusion protein: MRGSHHHHHH. The fusion protein was purified according to the manufacturer's instructions. Imidazole (300 mm) was used to elute $HrpZ_{Pss}$ protein, followed by extensive dialysis (3000-fold) in 5 mM $MgCl_2$ at 4°C. The purity of $HrpZ_{Pss}$ fusion protein was estimated by SDS–PAGE analysis to be greater than 95%. The fusion protein at the concentration of 80 μg ml^{-1} elicited a strong HR in tobacco and cucumber leaves, while an identical preparation from DH5α(pQE30) (used as a control in the purification) did not elicit any visible response in the same leaves.

Pseudomonas syringae strains were grown in King's B broth (King *et al.*, 1954) overnight at 30°C. Bacterial suspensions were prepared in 5 mM $MgSO_4$. Spores of *Colletotrichum lagenarium* were prepared as described previously (Kuc and Richmond, 1977). Tobacco necrosis virus inoculum was prepared by grinding cucumber leaves bearing necrotic local lesions in water (1g infected leaf tissue per 10 ml distilled water).

Induction of SAR

First true leaves (Leaf 1) of young cucumber plants (cv. 'Marketer') were treated with test agents by infiltration through their abaxial surfaces at 30 sites per leaf, with 10 μl per site delivered by a repeating pipettor. Treatments consisted of buffer (5 mM $MgSO_4$), $HrpZ_{Pss}$ (final concentration in buffer was 80–160 μg ml^{-1}), Pss61 or *hrpH* (a final OD_{600}=0.2 in 5 mM $MgSO_4$, equivalent to approximately 2×10^8 cells ml^{-1}), or a spore suspension of *C. lagenarium* (7.5×10^4 spores ml^{-1}).

For experiments involving tobacco (*Nicotiana tabacum* Samsun NN) plants, the third and fourth true leaves of 6-week-old plants were inoculated with TMV (100–150 lesions per leaf) or infiltrated with 120 μg ml^{-1} $HrpZ_{Pss}$ or 5 mM $MgSO_4$. For TMV inoculation, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TMV suspension. For inoculation with $HrpZ_{Pss}$ or 5 mM $MgSO_4$, 50 μl solution was

pressured into each of 10 panels of a tobacco leaf using a needleless syringe. Five plants were used for each treatment.

Assessment of SAR

At 7–8 days after treatment of Leaf 1 with test agents, subsequently developed leaves (usually Leaf 2 and/or Leaf 3) were challenged with *C. lagenarium*, TNV or *P. s.* pv. *lacrymans*.

For fungal challenge, 20 sites per leaf received 10 µl droplets of a *C. lagenarium* spore suspension (1×10^5 spores ml^{-1}) placed on adaxial surfaces with a repeating pipettor. After inoculation, plants were held in darkened moist chambers for 24 h to facilitate penetration of leaves by the pathogen. Chambers were then gradually opened to allow plant adaptation to ambient conditions over a 12 h period, and plants were then returned to a greenhouse bench for an additional 6–7 days to allow disease development.

For TNV challenge, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TNV suspension. Virus-inoculated plants were maintained on a greenhouse bench for 8–10 days to permit disease development.

For assessment of SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*, Leaf 1 was infiltrated with buffer, *C. lagenarium*, or $HrpZ_{Pss}$ as described above, and Leaf 5 was challenged on the abaxial surface with the bacterium by spraying with a bacterial suspension ($OD_{600}=0.1$) containing 0.02% Silwet L-77, a surfactant, at 11 days post-induction or by rubbing with a cheesecloth pad saturated with a bacterial suspension ($OD_{600}=0.2$) at 17 days after induction treatment. Spray-inoculated leaves were misted once and plants were then placed in a darkened moist chamber for 18 h, followed by a 12 h acclimation period. Plants were subsequently returned to the greenhouse bench. Rub-inoculated leaves were misted once with water and plants were kept on a greenhouse bench. Disease was allowed to develop for 7 days for rub-inoculated plants or 13 days for spray-inoculated plants.

For evaluation of anthracnose development, the number and diameter of necrotic lesions caused by *C. lagenarium* were determined, and the total necrotic area per leaf was calculated. The extent of disease caused by TNV or *P. s.* pv. *lacrymans* was evaluated by counting necrotic local lesions on entire inoculated leaves.

For assessment of SAR to TMV, the seventh and eighth true leaves were challenge-inoculated with TMV (100–150 lesions per leaf) 5 days after induction. For each treatment the diameters of 100 TMV lesions (from 10 leaves of five plants) were recorded.

PR protein assay

Tissues were collected from Leaf 1 and Leaf 2 during the 14 day period following induction of Leaf 1. The leaf tissues were rapidly frozen with dry ice and stored at −80°C. Protein extraction was based on the method previously described (Ji and Kuc, 1995). Frozen leaf tissues were homogenized at 0–4°C in 0.1 M sodium citrate buffer, pH 5.4, containing 0.1% (v/v) β-mercaptoethanol and 0.1% (w/v) L-ascorbic acid. The homogenate was centrifuged at 12 000 *g* for 30 min. The supernatant was decanted and dialyzed against two changes of water for 24 h and then against two changes of 0.05 M sodium acetate buffer (pH 5.0) for 2 h. The extract was centrifuged again at 10 000 *g* for 10 min. The supernatant was used as crude enzyme extract. Protein concentrations were measured using the Bio-Rad protein assay kit with bovine gamma globulin as standard.

Determination of enzyme activities in cucumber leaves

Protein patterns and peroxidase isozymes were analyzed after a single separation using a 15% (w/v) native-PAGE gel (Pan *et al.*, 1989). Peroxidase activity was determined using guaiacol as substrate (Hammerschmidt *et al.*, 1982). β-1,3-glucanase and chitinase activities were detected as described elsewhere (Ji and Kuc, 1995).

Expression of pr-1 gene in tobacco leaves

An internal fragment (from nt 304 to 535) of the tobacco *pr*-1 gene (Figure 1 in Cornelissen *et al.*, 1986) was amplified in a polymerase chain reaction (PCR) and labeled with [α-^{32}P]-dATP. Total RNA was purified from systemic leaves (the ninth true leaves) of tobacco plants 5 days post-induction. Ten micrograms of RNA from each treatment were fractionated in a 1.2% agarose/formaldehyde gel and subsequently blotted to Immobilon-N membrane (Millipore). Hybridization was performed in a solution consisting of 6×SSC, 2×Denhardt's reagent, 0.1% SDS and 10% dextran sulfate at 55°C. Washes were carried out in 0.2×SSC, 0.1% SDS at 60°C.

Acknowledgements

We wish to thank Doug Brown for growing plants, J. Shaw and G. deZoeten for providing us with TMV, David Smith, Wensheng Wei, Qian Yong, and Jing Yuan for critical review of the manuscript, and Karen Bird for help in manuscript preparation. This work was supported by grants from USDA/NRICGP (93-37303-9385) and DOE (DE-FG02-91ER20021).

References

Ariat, M., van Gijsegem, F., Heut, J.C., Pernollet, J.C. and Boucher, C.A. (1994) PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* **13**, 543–553.

Baker, C.J., Orlandi, E.W. and Mock, N.M. (1993) Harpin, an elicitor of the hypersensitive response in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells. *Plant Physiol.* **102**, 1341–1344.

Cameron, R.K., Dixon, R.A. and Lamb, C.J. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* **5**, 715–725.

Chen, Z., Silva, H. and Klessig, D.F. (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science*, **262**, 1883–1886.

Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M., Van Loon, L.C. and Bol, J.F. (1986) Molecular characterization of messenger RNAs for 'pathogenesis-related' proteins 1a, 1b, and 1c, induced by TMV infection of tobacco. *EMBO J.* **5**, 37–40.

Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A. and Dangl, J. L. (1994) *Arabidopsis* mutants simulating disease resistance response. *Cell*, **77**, 565–577.

Fenselau, S., Balbo, I. and Bonas, U. (1992) Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant–Microbe Interact.* **5**, 390–396.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, **261**, 754–756.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol.* 20, 73–82.

He, S.Y., Huang, H.-C. and Collmer, A. (1993) *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell*, 73, 1255–1266.

He, S.Y., Bauer, D.W., Beer, S.V. and Collmer, A. (1994) The hypersensitive response elicited by the *Erwinia amylovora* harpin requires active plant metabolism. *Mol. Plant–Microbe Interact.* 7, 289–292.

Huang, H.-C., Hutcheson, S.W. and Collmer, A. (1991) Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and TnphoA tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol. Plant–Microbe Interact.* 4, 469–476.

Huang, H.-C., He, S.Y., Bauer, D.W. and Collmer, A. (1992) The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: an envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* 174, 6878–6885.

Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A. and Deng, W.-L. (1995) The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. *Mol. Plant–Microbe Interact.* 8, 733–746.

Jakobek, J.L. and Lindgren, P.B. (1993) Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. *Plant Cell*, 5, 49–56.

Ji, C. and Kuc, J. (1995) Purification and characterization of an acidic β-1,3 -glucanase from cucumber and its relationship to systemic induced disease resistance induced by *Colletotrichum lagenarium* and tobacco necrosis virus. *Mol. Plant–Microbe Interact.* 8, 899–906.

Keen, N. T., Erwin, T., Long, M., Brugger, B. and Holliday, M. (1981) Inhibition of the hypersensitive response of soybean leaves to incompatible *Pseudomonas* spp. by blasticidin S, streptomycin and elevated temperature. *Physiol. Plant Pathol.* 18, 325–337.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of phycocyanin and fluorescin. *J. Lab. Clin. Med.* 44, 301–307.

Klement, Z. (1982) Hypersensitivity. In *Phytopathogenic Prokaryotes*, Volume 2 (Mount, M.S. and Lacy, G.H., eds). New York: Academic Press, pp. 149–177.

Kuc, J. (1982) Induced immunity to plant disease. *BioScience*, 32, 854–860.

Kuc, J. and Richmond, S. (1977) Aspects of the protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology*, 67, 533–536.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic-acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science*, 250, 1002–1004

Metraux, J.-P., Signer, H., Ryals, I., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, 250, 1004–1006.

Metraux, J.-P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant–Microbe Interactions*, Volume 6 (Hennecke, H. and Verma, D.P.S., eds). Dordrecht, Kluwer Academic Publishers, pp. 432–439.

Pan, S.Q., Ye, X.S. and Kuc, J. (1989) Direct detection of β-1,3-glucanase isozymes on polyacrylamide electrophoresis and isoelectrofocusing gels. *Anal. Biochem.* 182, 136–140.

Preston, G., Huang, H.-C., He, S.Y. and Collmer, A. (1995) The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not in soybean. *Mol. Plant–Microbe Interact.* 8, 717–732.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 97, 1342–1347.

Ross, A.F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology*, 14, 340–358.

Sequeira, L. (1979) Bacterial hypersensitivity. *Nicotiana*: procedure for experimental use. *USDA Tech. Bull.* 1588, 111–120.

Smith, A. S., Hammerschmidt, R. and Fulbright, D.W. (1991) Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol.* 38, 223–235.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E. and Ryals, J. (1993) Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant–Microbe Interact.* 6, 692–698.

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P. and Boucher, C. (1995) The *hrp* locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15, 1095–1114.

Wei, Z.-M. and Beer, S.V. (1993) Hrpl of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. *J. Bacteriol.* 175, 7958–7967.

Wei, Z.-M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A. and Beer, S. V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science*, 257, 85–88.

White, R. F. (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, 99, 410–412.